Biosynthesis of (2*E*,4*E*,6*E*)-5-Ethyl-3-Methyl-2,4,6-Nonatriene: the Aggregation Pheromone of *Carpophilus freemani* (Coleoptera: Nitidulidae)*

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The biosynthesis of the male-produced aggregation pheromone of *Carpophilus freemani* Dobson (Coleoptera: Nitidulidae) was studied by feeding the beetles on diet containing organic acids labeled with ^{2}H (acetate, propionate, and butyrate) or ^{13}C (acetate). The emitted pheromone, (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene, was collected and analyzed by mass spectrometry and NMR spectrometry to determine the positions of isotopic labels. Studies with four deuterium-labeled synthetic pheromone standards demonstrated that mass spectrometry would be useful in locating the labels in beetle-derived samples. The pheromone is built up from one acetate unit, one propionate unit, and then two butyrate units, accompanied by the loss of the carboxyl carbon from one of the butyrates. The beetles will use propionic and butyric acid if it is available in the diet, but they are capable of making these acyl units from acetate if necessary. Biosynthetic steps for removing the acyl oxygens and making the double bonds of the pheromone are proposed to be as in usual fatty acid anabolism (reductions and dehydrations).

Pheromone   Attractant   Biosynthesis   Mixed-origin polyketide   Butyrate   Stable isotopes   Triene
Hydrocarbon   Insecta   Coleoptera   Nitidulidae   *Carpophilus freemani*

INTRODUCTION

A series of unusual triene and tetraene hydrocarbons have recently been identified as aggregation pheromones in sap beetle species of the genus, *Carpophilus* (Coleoptera: Nitidulidae). The pheromones are male-produced but attract beetles of both sexes (Bartelt *et al.*, 1992 and references therein). All of these compounds have their alkyl branches on alternate carbons and have a conjugated olefinic system. Example structures are shown in Fig. 1 (1–5).

Pheromones with related carbon skeletons are known for a number of other beetle species such as *Lasioderma serricorne* F. and *Stegobium paniceum* L. (both Anobiidae) (see review by Vanderwel and Oehlschlager, 1987). These pheromones also have the branch points on

alternate carbons but differ from the *Carpophilus* pheromones in being oxygenated and in having fewer double bonds [Fig. 1 (6–7)]. Chuman *et al.* (1983) pointed out that the pheromone components of *L. serricorne*, including 7, fit the branching and oxygenation pattern of a polyketide, and that assembly of the chains from propionate units, accompanied by a decarboxylation would account nicely for the carbon skeletons. The *Carpophilus* pheromones could similarly be assembled from simple acyl units, but precursors in addition to propionate would be required (Bartelt *et al.*, 1992). Note that the carbon skeletons of 4 and 6 are identical, and the same is true for 5 and 7.

Despite speculation about biosynthesis of these beetle pheromones, there has been no experimental verification of any of these routes. We chose to study pheromone biosynthesis in *C. freemani* Dobson as one example of a polyketide pheromone. This species is easily reared and produces a large amount of pheromone, especially 1 (Fig. 1). The origin of the ethyl branch was of particular interest because these can originate in natural compounds in a number of ways: from propionate (insect juvenile hormone JHII, Schooley *et al.*, 1973), by C-methylation of an existing methyl group (barnol, a *Penicillium* metabolite, Better and Gatenbeck, 1977), or
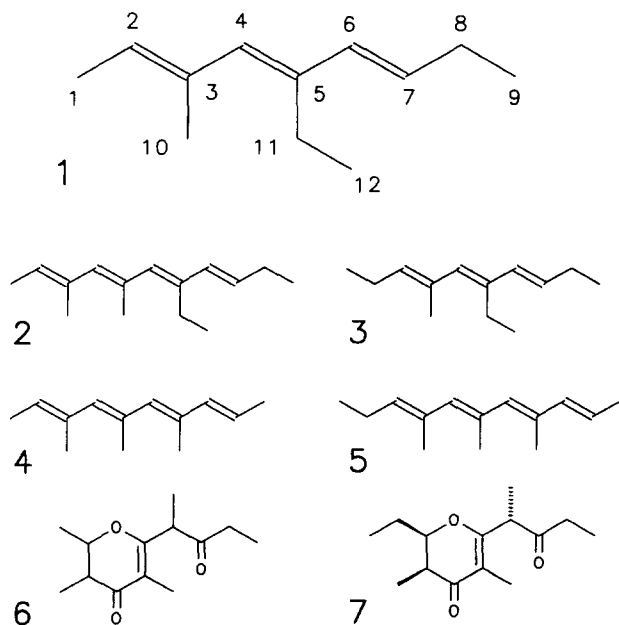
FIGURE 1. Pheromones of proposed polyketide origin. The compounds (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene (1), (2*E*,4*E*,6*E*, 8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-undecatetraene (2) and (3*E*,5*E*,7*E*)-6-ethyl-4-methyl-3,5,7-decatriene (3) are from *C. freemani*. The compounds (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene (4) and (2*E*,4*E*,6*E*,8*E*)-4,6,8-trimethyl-2,4,6,8-undecatetraene (5) are male-produced aggregation pheromones from *C. hemipterus*. Stegobinone (6) is the sex pheromone produced by the female drugstore beetle, *S. paniceum* (Kuwahara *et al.*, 1978) and (2,3-*cis*)-serricorone (7) is a sex pheromone component of the cigarette beetle, *L. serricorne* (Chuman *et al.*, 1983).

by intact incorporation of butyrate (antibiotic X-537A, Westley *et al.*, 1970). We had previously suggested butyrate incorporation for the *C. freemani* pheromone (Bartelt *et al.*, 1990c) but did not have experimental evidence.

Here we report the biosynthetic origins of all carbons in the *C. freemani* pheromone. The method was to analyze emitted pheromone after beetles had fed on a diet containing precursors labeled with ^{2}H or ^{13}C. Originally, the intent was to monitor label incorporation by mass spectrometry. Four deuterium-labeled standards were prepared to clarify the fragmentation mechanism of the pheromone, and we found that mass spectrometry can be used to locate the heavy-isotope label within the molecule fairly successfully. However, the beetles produced pheromone in high enough quantities and with high enough isotopic enrichment that NMR spectrometry could also be used for analysis, allowing label locations to be determined even more precisely.

MATERIALS AND METHODS

Beetle culture

The *C. freemani* culture was the same one used earlier for pheromone identification (Bartelt *et al.*, 1990c). The artificial diet was described previously (Dowd, 1987), except that the pinto beans were replaced by an equal weight of brewer's yeast.

Diets with labeled precursors

All labeled precursors were purchased from Cambridge Isotope Laboratories, Woburn, Mass. 20 ml of diet was prepared for each experiment using the following precursors (percentage in diet given in parentheses): 3,3,3-D$_3$-propionic acid (1%), 3,3,4,4,4-D$_5$-butyric acid (1%), 1,2,2,2-D$_4$-acetic acid (5%), 1-[^{13}C]acetic acid (5%), or 2-[^{13}C]acetic acid (5%). Precursors were added to a hot diet and thoroughly mixed in a Waring blender.

Unexpectedly, the deuterium-labeled butyrate contained 2,3,3,4,4,4-D$_6$-, and 2,2,3,3,4,4,4-D$_7$- species in addition to the D$_5$-butyrate. Ratios of D$_5$-, D$_6$- and D$_7$-species were estimated to be 1:2:1, respectively, based on intensities of the molecular ions at m/z 93, 94 and 95, corrected for the natural abundance of ^{13}C. (The formula weight of unlabeled butyric acid is 88.) Fragments for 3,3,4,4,4-D$_5$-butyrate were expected at m/z 61 and 75, but additional intense fragments were also seen at m/z 62, 63, 76, and 77. Analogous additional fragments were obtained for the methyl ester of the acid (formed with diazomethane). There was an intense peak for the ester at m/z 59, but not at 60, which indicated the CH$_3$-O-(CO)-$^+$ fragment was not labeled. Therefore, all of the additional label in the free butyrate was at C-2 but not at the carboxyl group.

Pheromone collections

Freshly emerged beetles were immobilized by chilling over ice and separated by sex. Approximately 200–400 male *C. freemani* were placed in each 50-ml collection flask with *c.* 10 ml of artificial diet. As the food began to dry out (after about 7 days), additional diet containing the labeled precursor was added (5–10 ml). Each experiment lasted 2–4 weeks. The volatiles emitted by feeding beetles were collected onto porous polymer as described previously (Bartelt *et al.*, 1990c) except that Super Q (Alltech Associates, Deerfield, Ill.) was used instead of Tenax. Super Q volatile-collection traps were extracted every 2 or 3 days by rinsing with 500 μl of hexane. Hydrocarbons were obtained from volatile collections by silica gel chromatography on open columns using pentane as the eluant. Each pheromone collection was analyzed by GC and GC–MS. All collections for a given labeled precursor were eventually combined and analyzed by NMR. (The pheromone samples were suitable for NMR after the single pass through silica gel; purities were >80% and no other constituent exceeded 3%.)

Analysis of pheromone collections

Pheromone collections were monitored by gas chromatography (GC) using a Varian 3700 GC, equipped with a 15 m × 0.25 mm i.d. DB-1 capillary column (1.0 μm film thickness, J&W Scientific, Rancho Cordova, Calif.). The instrument was interfaced to a Hewlett-Packard 3396A integrator. The temperature program was 100–200°C at 10° per min. Pheromone production was quantitated relative to an internal standard (*n*-heptadecane).

TABLE 1. [^{13}C]- and [^{1}H]NMR data[a] for compound 1

Position	C[b]	Number of attached protons	H[c] (mult., J)
1	13.9	3	1.63 (br d, 1.0, 6.8)
2	124.0	1	5.99 (dqq, 1.4, 2.8, 6.8)
3	134.1	0	—
4	133.5	1	5.96 (br s)
5	139.2	0	—
6	133.9	1	6.10 (br d, 15.7)
7	129.9	1	5.74 (dt 15.7, 6.6)
8	26.5	2	2.10 (ddq, 1.2, 6.6, 7.5)
9	14.2	3	1.02 (t, 7.5)
10	16.7	3	1.77 (br s)
11	21.1	2	2.52 (q, 7.5)
12	14.9	3	1.18 (t, 7.5)

[a] [^{1}H]- and [^{13}C]NMR spectra were recorded in C_6D_6. Shifts are reported in ppm relative to TMS.

[b] C_6D_6 as internal reference (128 ppm). Numbers of attached protons confirmed with DEPT experiments. Assignments based on 2D NMR.

[c] Residual C_6D_5H as internal reference (7.20 ppm). Coupling constants are given in Hz.

Electron-impact mass spectra of beetle-derived and synthetic compounds were obtained on a Hewlett–Packard 5970 mass selective detector. Sample introduction was through a Hewlett–Packard 5890A gas chromatograph fitted with a 15 m DB-1 capillary column.

Proton and [^{13}C]NMR spectra were recorded on a Bruker WM 300 MHz WB spectrometer. All samples were dissolved in C_6D_6. Chemical shifts are reported in parts per million (ppm) from tetramethylsilane with the residual proton resonance of C_6D_5H as the internal reference for protons (7.20 ppm) and the center ^{13}C resonance of C_6D_6 as an internal reference for ^{13}C (128 ppm). The same 5 mm ^{1}H/^{13}C dual probe was used for both proton and carbon observation.

Assignment of ^{13}C resonances for the C. freemani pheromone

The proton NMR spectrum was previously assigned for compound 1 (Bartelt et al., 1990c), but the ^{13}C spectrum and carbon assignments were needed as well for interpretation of the labeling experiments. The ^{13}C spectrum was acquired for synthetic 1. The heteronuclear correlation experiment (Bax and Morris, 1981) was used to assign the shifts for those carbons with attached hydrogens. Assignment of the remaining quaternary carbons (3 and 5) was by analogy to previously assigned tetraenes with relevant partial structures (Bartelt et al., 1990a). The DEPT experiment (Doddrell et al., 1982) was used to confirm the proton multiplicity of each carbon. The NMR properties of the unlabeled pheromone are summarized in Table 1.

Synthesis of deuterated pheromone standards

In order to use mass spectrometry to determine the location of isotope labels in the pheromone molecule, it was essential to understand the origins of key fragment ions. This was accomplished by synthesizing four deuterated standards 1a–d (Fig. 2) and analyzing their mass spectra. These standards had deuterium at four locations expected to become labeled during the feeding experiments.

The general scheme for preparing the standards is given in Fig. 2. Wittig and Wittig–Horner reactions were the key steps. Reaction conditions were generally as described by Bartelt et al. (1990a, 1992). The deuterated Wittig and Wittig–Horner reagents were prepared from deuterated alkyl iodides and then used without intermediate isolation.

The final products were purified by silica gel chromatography on open columns, then by HPLC on AgNO$_3$-coated silica and size-exclusion columns (Bartelt et al., 1990b). Amounts purified for spectral analysis were 100–500 μg, and all purities were >80%, by GC. This purity was sufficient to allow verification by NMR that the labels were in the proper locations; the GC inlet of the mass spectrometer further separated the labeled compounds from impurities so that the spectra of the pure compounds could be obtained. The proton NMR spectra of the standards completely supported the previous resonance assignments (Bartelt et al., 1990c). As expected, signals for the deuterated sites were generally absent. The exception was 1c, in which the signals for the protons at C-11 and -12 were about 20% of the maximum (undeuterated) intensity instead of being completely absent. In the synthesis of 1c, the triethyl 2-phosphonoacetate reagent was apparently alkylated to some extent by the ester ethyl groups of other reagent molecules as well as by the labeled ethyl iodide. The mass spectrum of pure 1c was nevertheless obtainable because the deuterated compound eluted from the GC column slightly before the unlabeled product. The key mass spectral peaks for all of the labeled standards are given in Table 2.
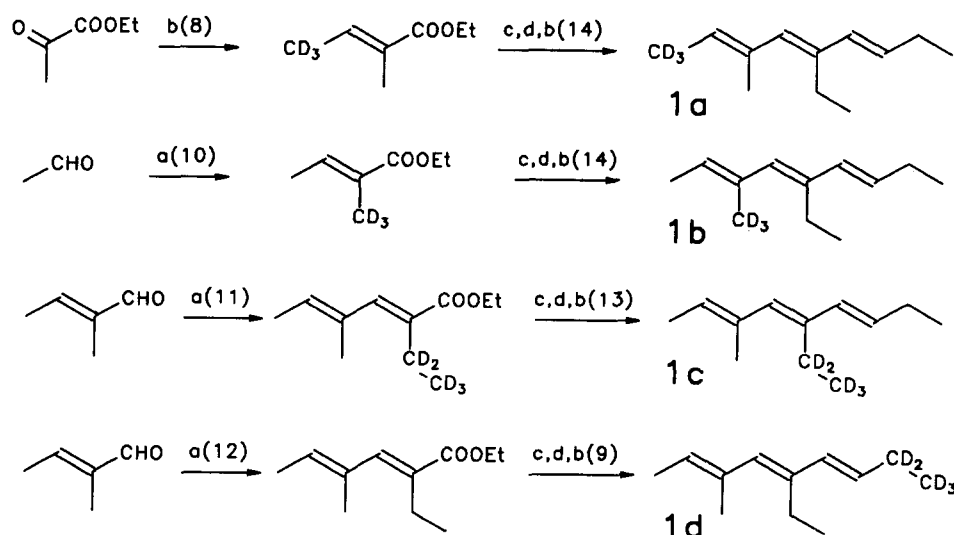
RESULTS

Label location by mass spectrometry

From the spectra of the deuterium-labeled standards, 1a–d (Table 2), the origins of several major fragment ions in the mass spectrum of 1 were defined (Fig. 3). This information allowed the locations of deuterium atoms to be determined in many of the beetle-derived pheromone samples from the labeled-precursor experiments. The deuterium-labeled and unlabeled pheromone species were separable by GC, allowing the mass spectra of the labeled species to be seen without interference.

In all cases, the mass spectra indicated the overall degree of label incorporation because the molecular ion was prominent. The base peak (m/z 107 in the unlabeled pheromone) was one good indicator of label location in deuterated pheromones. If the base ion was unlabeled or had, at most, one deuterium, then the pheromone was labeled in the ethyl branch (C-11 and -12) or ethyl terminus (C-8 and -9); both ethyl groups are cleaved off when the base ion forms (Fig. 3). The major mechanism that forms the base ion involves a hydrogen (or deuterium) transfer from the ethyl branch; thus the base

SYNTHETIC ROUTES TO DEUTERATED STANDARDS

O COOEt   b(8)   CD₃ COOEt   c,d,b(14)   CD₃ **1a**

CHO   a(10)   COOEt CD₃   c,d,b(14)   CD₃ **1b**

CHO   a(11)   COOEt CD₂CD₃   c,d,b(13)   CD₂CD₃ **1c**

CHO   a(12)   COOEt   c,d,b(9)   CD₂CD₃ **1d**

INTERMEDIATES

CH_3Ph_3PI $\xrightarrow[RI]{BuLi}$ $R-CH_2Ph_3PI$

8 : R = -CD₃
9 : R = -CD₂CD₃

$EtOCCH_2P(OEt)_2$ $\xrightarrow[RI]{BuLi}$ $EtOCCHP(OEt)_2$

10 : R = -CD₃
11 : R = -CD₂CD₃

$EtOCCHP(OEt)_2$ Et   **12**
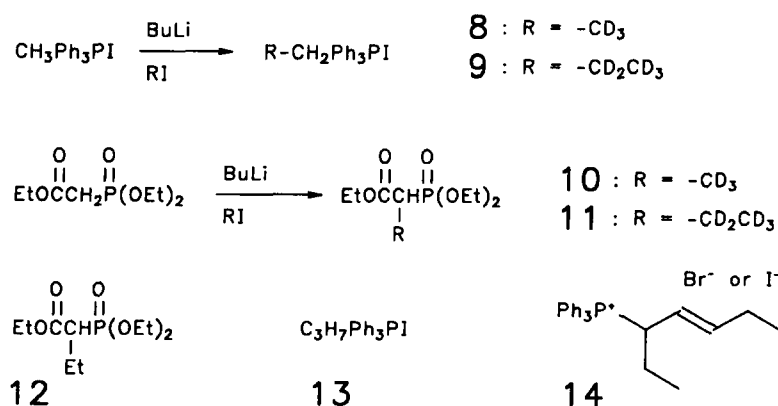
$C_3H_7Ph_3PI$ **13**

Ph_3P^+ Br⁻ or I⁻   **14**

FIGURE 2. Syntheses of deuterated standards. a: Wittig–Horner reaction (reagent in parentheses); b: Wittig reaction (reagent in parentheses); c: LiAlH₄ reduction to corresponding alcohol; d: MnO₂ oxidation of alcohol to aldehyde. Intermediates 8–11 were prepared and used in one pot.

peak is m/z 108 if C-11 and -12 are deuterated. The single cleavage fragments at m/z 135 and 140 further indicate which of the two ethyl groups is deuterated because the terminal ethyl is lost more readily than the ethyl branch (Fig. 3; compare 1c and 1d in Table 2). If the molecular ion and base peak both indicate the same degree of labeling, then the label is not at C-8, -9, -11, or -12.

Unfortunately, a label at C-1 is not readily distinguished from a label at C-10 by mass spectrometry because both methyl groups are lost about equally readily (Fig. 3). There were subtle differences in the fragment intensities at m/z 91–96 for 1a and 1b, but these were not sufficient for reliable differentiation of C-1 and -10.

Pheromone production

By GC, total production of pheromone by beetles feeding on labeled diets was as follows: 0.34 mg with 3,3,3-D₃-propionate, 0.24 mg with 3,3,4,4,4-D₅-butyrate,

0.17 mg with 1,2,2,2-D₄-acetate, 1.84 mg with 1-[¹³C]acetate, and 1.50 mg with 2-[¹³C]acetate. Typical production of 1 was 100–250 ng per beetle per day.

Incorporation of 3,3,3-D₃-propionate

By mass spectrometry, there were two major pheromone species in the volatile collections, unlabeled and trideuterated (indicated by molecular ions at m/z 164 and 167, respectively). Over the six collections, the trideuterated species ranged from 26 to 49% of the total pheromone (mean = 35%). The incorporation was highest with fresh diet and declined as the diet dried out. From the relative abundances of the molecular ions, taking into consideration the natural abundance of ¹³C, the unlabeled and trideuterated forms accounted for 93% of the total pheromone. Thus, there was very little scrambling of the label, and the trideuterated methyl group was incorporated essentially intact.

TABLE 2. Intensities[a] of key mass spectral ions for compound 1 and synthetic deuterated analogs (1a–d)

m/z	Intensity				
	1	1a	1b	1c	1d
Molecular ion					
164	13	—	—	—	—
167	—	16	12	—	—
169	—	—	—	18	18
Loss of methyl					
149	5.7	1.5	1.0	—	—
151	—	—	—	2.9	1.1
152	—	4.3	4.0	0.5	0.3
154	—	—	—	4.2	6.8
Loss of ethyl					
135	41	0.9	0.2	12	40
138	—	44	39	0.8	0.3
140	—	0.5	0.2	47	12
Cluster around base peak					
107	100	9	8	24	100
108	10	12	13	100	25
109	2	—	—	22	6
110	—	100	100	6	3
111	—	9	9	5	3
112	—	0.4	0.5	7	3
Masses 91–96					
91	39	16	15	17	22
92	6	7	7	19	11
93	25	19	16	15	15
94	2	14	14	23	18
95	—	4	4	9	6
96	—	9	15	4	0

[a] Intensities expressed as percent of base peak.

The mass spectrum of deuterated pheromone had a base peak of m/z 110, thus the label was not at C-8, -9, -11, or -12. The NMR spectrum of the combined pheromone collections confirmed that the label was located on C-10 (Fig. 4). The proton signal for this methyl group had an area 30% less than in the spectrum of unlabeled 1, in good agreement with the incorporation calculated from the mass spectra.

*Incorporation of 3,3,4,4,4-D5-butyrate (and more heavily labeled butyrates)*

Five major species were observed in the volatile collections (percent of total pheromone given in parentheses, correcting for natural abundance of ^{13}C): D_0 (32%), D_5 (25%), D_6 (9%), D_{10} (10%), and D_{11} (8%). Molecular ions for these species were m/z 164, 169, 170, 174, and 175, respectively. Together, these accounted for 84% of the total pheromone. As in the propionate feeding, label incorporation was highest when the diet was fresh.

Interpretation of these results was more complex because the labeled butyrate contained 2,3,3,4,4,4-D_6-butyrate (50%) and 2,2,3,3,4,4,4-D_7-butyrate (25%) in addition to the intended 3,3,4,4,4-D_5-butyrate (25%). The molecular ions at m/z 169 and 174 would be expected with intact incorporation of one and two D_5-butyrates, respectively. For pheromone with molecular ion m/z 174, the base peak was m/z 108, and m/z 140 (having 5 of the 10 deuteriums) was the only fragment

in the spectrum related to loss of an ethyl group. This pattern indicated that all of the label was at C-8, -9, -11, and -12 (the terminal and branch ethyl groups). For pheromone with molecular ion m/z 169, the peaks at m/z 107 and 108 were similar in intensity, as were those at m/z 135 and 140. Two pheromone species were probably present, not separable by GC: D_5-C-8/C-9 and D_5-C-10/C-11.

We believe that the m/z 170 and 175 ions would not have been observed if the D_6- and D_7-butyrates were not in the diet. These pheromone species exhibited the same fragmentation patterns as the D_5- and D_{10}-pheromone except that base peaks and key fragments were increased by one mass unit (i.e. m/z 109 was observed instead of 108, and 136 and 141 were observed instead of 135 and 140). Therefore, one additional deuterium was present at a location other than at C-8, -9, -11, or -12.

By proton NMR, the methyl (δ 1.02 and 1.18) and methylene (δ 2.10 and 2.52) signals for the ethyl terminus and ethyl branch were reduced by 17% ± 5%, compared with the unlabeled pheromone (Fig. 4). This supported the mass spectral evidence both qualitatively and quantitatively. In addition, the olefinic signal at γ 5.74 was also reduced by 8%. Therefore, the additional deuterium noted by mass spectrometry was located at carbon 7.

LOSS OF METHYL (m/z = 149)

LOSS OF ETHYL (m/z = 135)

BASE PEAK (m/z = 107)

18% OTHER MECHANISMS
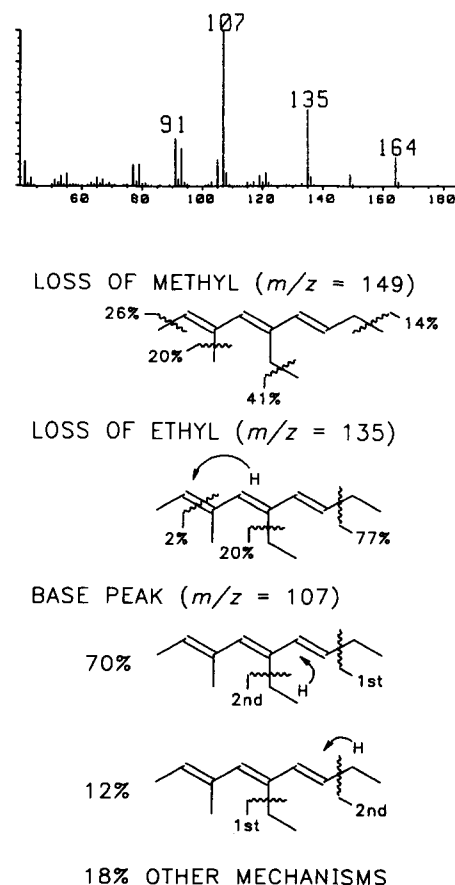
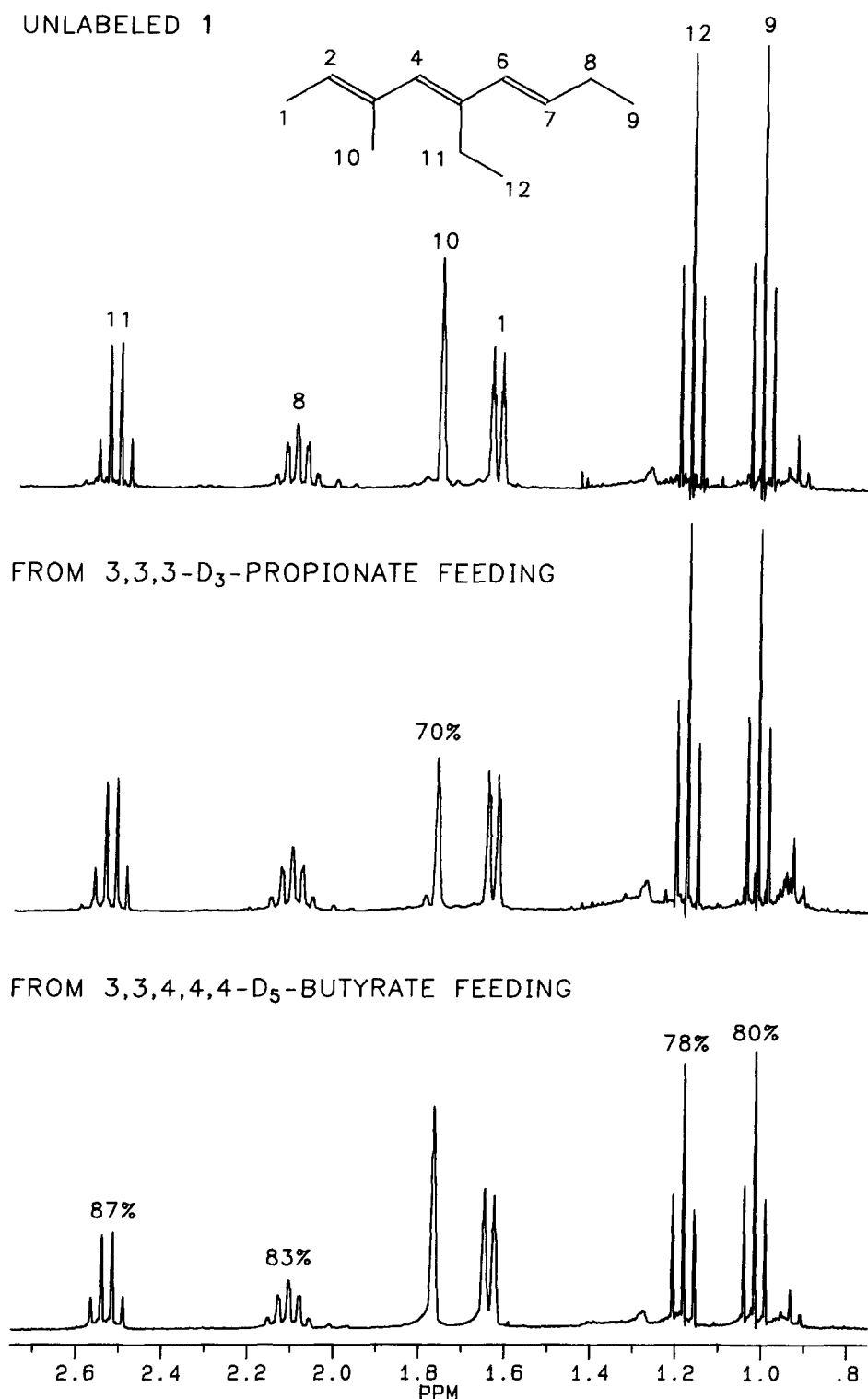FIGURE 3. Origins of several major fragment ions in the mass spectrum of 1, the aggregation pheromone of *C. freemani*.

FIGURE 4. [¹H]NMR spectra of unlabeled 1 and 1 derived from feeding beetles $CD_3CH_2CO_2H$ or $CD_3CD_2CH_2CO_2H$.

Incorporation of 1,2,2,2-D₄-acetate

By mass spectrometry, species with as many as nine deuteriums were detected, but the intensities of the ions decreased regularly from 31 to 0.01% of the intensity of m/z 164 as m/z increased from 165 to 173. The acetate methyl group was not incorporated intact, and the deuterium label appeared thoroughly scrambled. The NMR spectrum did not differ from that of the unlabeled

pheromone. The deuteroacetate feeding gave no information about pheromone biosynthesis. Therefore, the experiment was repeated with ¹³C-labeled acetates.

Incorporation of 1-[¹³C]acetate

Pheromone molecules having 0, 1, 2, 3 and 4 ¹³C atoms from 1-[¹³C] acetate could be identified by mass spectrometry, displaying molecular ions at m/z 164–168;

relative abundances within this cluster were 100:77: 32:9:1.5. Mass spectrometry gave little information about the locations of acetate-derived ^{13}C atoms because there was little GC separation among the unlabeled and various labeled species. [^{13}C]NMR, however, showed clearly that the additional ^{13}C abundance was almost exclusively at C-2, -6, -8, and -11 (Fig. 5). All other carbon signals of 1 were also detected, however, suggesting some scrambling of the label had occurred; of the minor spectral peaks, that for C-1 was the most prominent. Among the other minor peaks, the signal for C-3 was more intense than those of C-4 or -10, whereas in the unlabeled reference spectrum, the signal for C-3 was weaker relative to C-4 and -10.

Incorporation of 2-[^{13}C]acetate

Pheromone molecules having 0, 1, 2, 3, 4 and 5 ^{13}C atoms incorporated from 2-[^{13}C]acetate could be identified by mass spectrometry, displaying molecular ions at m/z 164–169; relative abundances within this cluster were 100:37:24:23:9:4. By [^{13}C]NMR, the signals for C-1, -5, -7, -9, and -12 were particularly enhanced (Fig. 5), but those for all other carbons except C-3 were also detected. [The quaternary carbon at δ 134.1 (C-3)
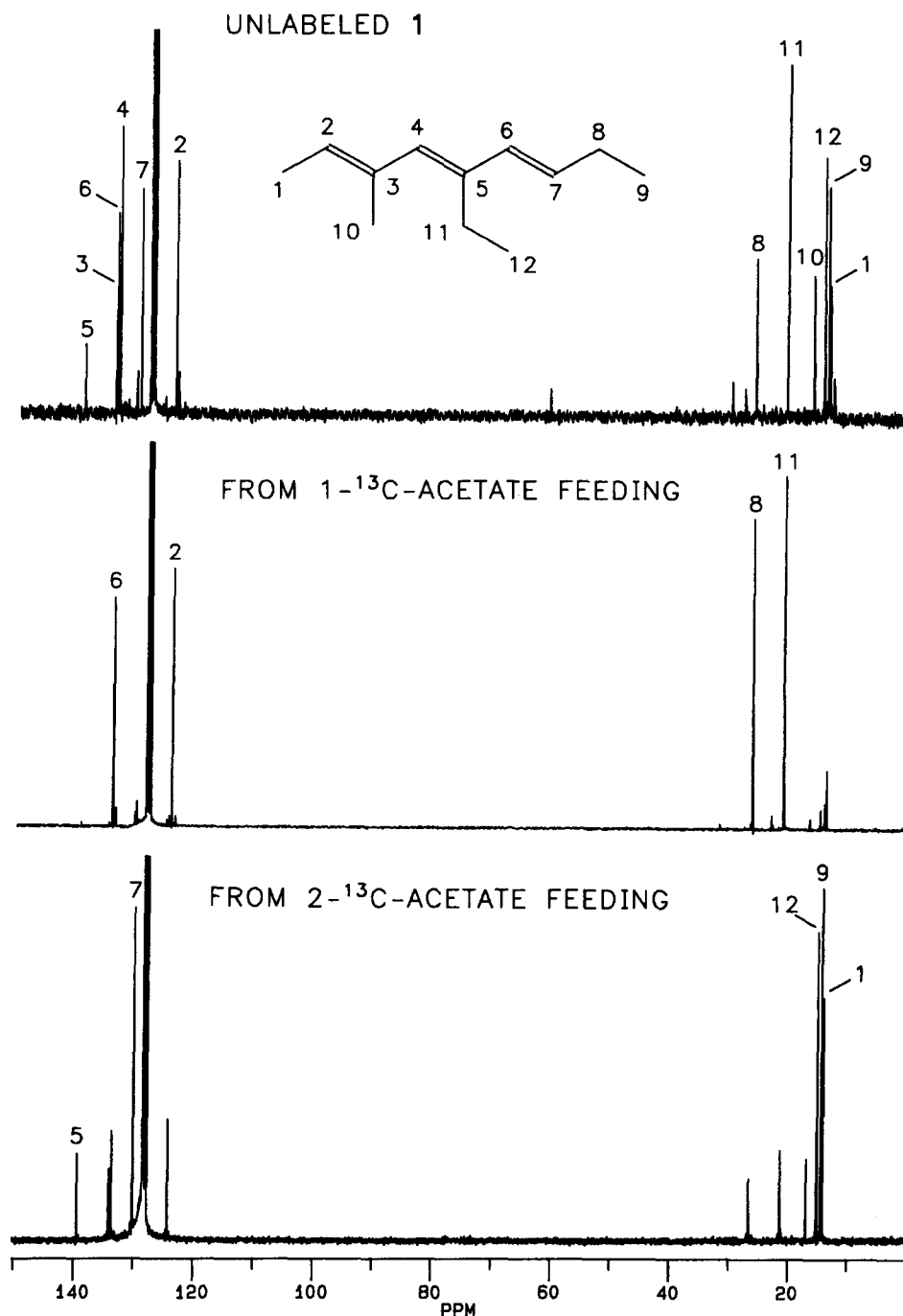

FIGURE 5. [^{13}C]NMR spectra of unlabeled 1 and 1 derived from feeding beetles [^{13}C]acetates. The highest peak in the spectrum is full scale.

was not seen, even when that portion of the spectrum was greatly expanded (not shown); the signal at δ 134.1 and its nearest neighbor, δ 133.9, were clearly resolved in other samples]. Label scrambling with 2-[¹³C]acetate was more extensive than with 1-[¹³C]acetate. Because of the relatively wide-spread labeling, carbon–carbon coupling by adjacent carbons was noticeable. No coupling was detected for C-10, however, which further supported the lack of label at C-3.

Minor pheromone components

These experiments provided evidence regarding *C. freemani* biosynthesis of triene 2 and tetraene 3, two minor constituents of its aggregation pheromone. Ingestion of 3,3,3-D₃-propionate led to incorporation of two labeled propionate units into both 2 and 3, as evidenced by prominent (M + 3)⁺ and (M + 6)⁺ peaks in the mass spectra. Furthermore, ingestion of 3,3,4,4,4-D₅-butyrate led to samples of 2 and 3 that gave (M + 5)⁺ ions in their respective mass spectra, indicating incorporation of one butyrate unit. No (M + 10)⁺ ions were observed in these mass spectra, but any (M + 10)⁺ ions probably would have been below the detection limit of the instrument for these minor components.

DISCUSSION

Pheromone production

It is worth noting that these experiments were possible only because the beetles were able to tolerate a very large amount of carboxylic acid in the diet (as much as 5% of the diet wet weight) and still produce good quantities of pheromone.

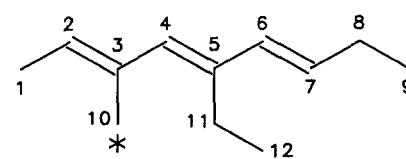#### Labeling patterns and pheromone biosynthesis

The observed labeling patterns, summarized in Fig. 6, are consistent with both patterns of acyl incorporation outlined in Fig. 7(A). From the ¹³C-labeling studies, C-1 and -2 of 1 are from acetate, with the carboxyl carbon of acetate becoming C-2. From the experiment with 3,3,3-D₃-propionate, the C-10 methyl group is from the terminal methyl of propionic acid. Because propionic acid is incorporated intact into 1, C-3 and -4 also arise from propionate. The fact that 1-[¹³C]acetate did not label these positions except at very minor levels is consistent with this conclusion.

The studies with ²H-labeled butyrate and ¹³C-labeled acetate indicated that C-5 through -9, -11, and -12 (seven total carbons) are derived from butyrate. The retention of label from 3,3,4,4,4-D₅-butyrate in both ethyl groups of 1 (C-8/C-9 and C-11/C-12) is strong evidence for its intact incorporation. Catabolism to acetate before incorporation would have resulted in complete loss of label in the ethyl methylene positions (C-8 and -11). The high degree of incorporation of labeled butyrate argues against other origins for the ethyl branch of the pheromone.
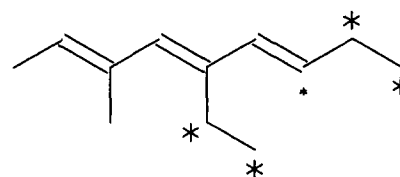
The additional information from (unintentional) incorporation of 2,3,3,4,4,4-D₆- and 2,2,3,3,4,4,4-D₇-

butyrates further supported the above conclusions. If the ethyl groups originate as described, then C-5 and -7 of 1 must come from the α carbons of the butyrates. Since C-5 has no protons and C-7 has only one, no more than one deuterium from the α position of butyrate can be incorporated per pheromone molecule, regardless of whether one or two labeled butyrate molecules ultimately become part of the pheromone. Thus, the existence of species with 170 or 175 mol. wt but not 171 or 176 from these more heavily labeled butyrates is readily explained, as is the reduced proton NMR signal for C-7.
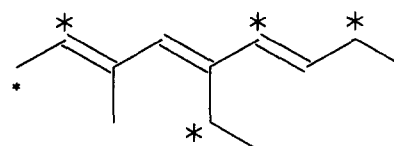
Our experiments did not determine definitely which of the two butyrate units loses its carboxyl carbon, but the D₆- and D₇-butyrates did provide some information about the decarboxylation. Removal of a carboxyl group from C-7 could not be the final biosynthetic step, as suggested previously (Fig. 5 in Bartelt *et al.*, 1990c), because no deuterium label could be present at C-7 of the
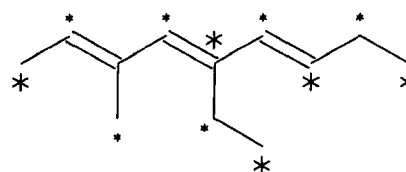

FIGURE 6. Summary of observed labeling patterns from feeding experiments. Major label incorporation was at positions labeled with *. Label was also incorporated into positions marked with a smaller asterisk (see text). In the 2-[¹³C]acetate feeding experiment, no label was incorporated into the C-3 position of 1.
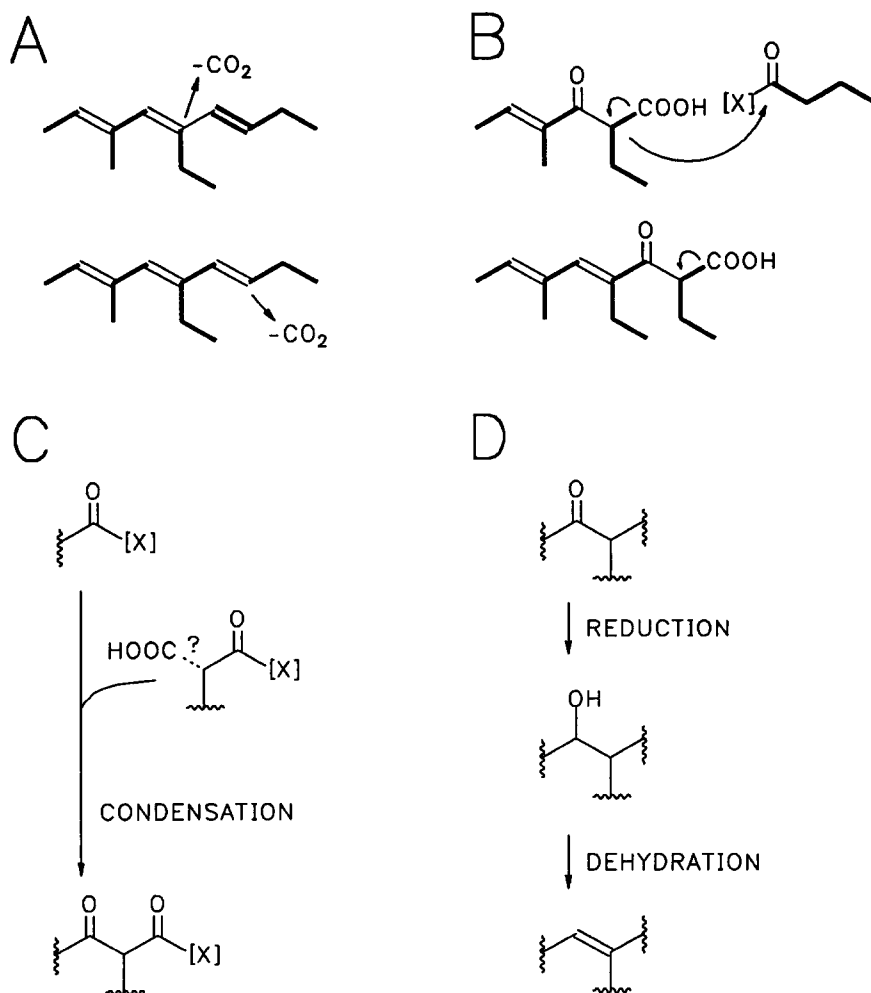
FIGURE 7. Summary of biosynthetic results: A: Origins of carbons in pheromone 1 from acetate, propionate, and butyrate acyl units. Heavy lines connect carbons from each acyl unit. It is not yet clear which of the butyrates loses its carboxyl group. B: possible intermediates in the decarboxylation step, corresponding to the patterns in A. C: acyl condensation reaction; acyl additions may be via alkylmalonates. D: reduction and dehydration reactions. [X] is an acyl carrier or hydroxyl group.

pheromone under that model. Instead, the decarboxylation must occur earlier in the biosynthesis, and two possibilities are shown in Fig. 7(B). It is likely that a β-keto intermediate is decarboxylated, as this sort of biochemical reaction is well known (e.g. conversion of acetoacetate to acetone, Lehninger, 1982, p. 525). The first alternative shown in Fig. 7(B) would lead to better retention of deuterium label at C-7 of the pheromone because the α hydrogens of butyrate are subjected to removal only during the dehydration step forming the double bond in the pheromone. In the second alternative, removal of an α hydrogen would occur during the condensation reaction that formed the figured intermediate as well as in the dehydration noted previously. With this alternative, survival of label at C-7 would depend on the stereospecificity of the reactions.

Overall, then, the chain is initiated with acetate, is elongated first with propionate then with butyrate, and is terminated with a second butyrate. The condensation reaction may be similar to that in Fig. 7(C); the acyl groups may be added as the corresponding alkyl-malonates. Removal of the carboxyl carbon of one

butyrate occurs before the final biosynthetic step [Fig. 7(B)]. Reduction and dehydration reactions [Fig. 7(D)] would remove the oxygens from the pheromone and create the double bonds. Such reactions are common in fatty acid anabolism. However, the sequence of these reactions relative to the condensations remains unknown.

Biosynthesis of butyrate and propionate from acetate and label scrambling

Label was incorporated into C-1 of 1 from the 2-[^{13}C]acetate feeding and into C-2 of 1 from the 1-[^{13}C]acetate feeding as hypothesized, but carbons that we established to be of butyrate origin were also labeled (C-6, -8, and -11 during the 1-[^{13}C]acetate feeding and C-5, -7, -9, and -12 during the 2-[^{13}C]acetate feeding). This is not surprising because acetate is a known precursor of butyrate. This finding does tell us that while the beetles will use butyrate if it is available in the diet, they are capable of making these four-carbon units from acetate if necessary.

The labeling by 2-[^{13}C]acetate was more complicated than with 1-[^{13}C]acetate. This is typical of biosynthetic studies with labeled acetate. Assimilation of acetate into an organism may be followed by direct conversion into a polyketide metabolite, or the labeled acid may be turned through the citric acid cycle before use in polyketide biosynthesis (Herbert, 1989, p. 34). When 1-[^{13}C]acetate enters the citric acid cycle, scrambling of label in the precursor does not occur, but some of the label is lost as carbon dioxide. However, when 2-[^{13}C]-acetate enters the citric acid cycle, acetate is subsequently released with some label on C-1 as well as on C-2. Thus feeding 2-[^{13}C]acetate can lead to a polyketide with label over all of its carbon atoms that would be labeled by actetate (Herbert, 1989, p. 34).

Interestingly, the 2-[^{13}C]acetate labeled C-4 and -10 of the pheromone, which are associated with propionate, but C-3, which is also from propionate, was not labeled. Carbon–carbon coupling was observed in the ^{13}C spectrum because the existence of label on two adjacent carbons was not improbable for this sample. Splitting was observed for all of the detectable signals except for C-10, which indicated in another way that C-3 was not labeled in the experiment. These results show that the three-carbon unit incorporated into the pheromone can also be constructed from acetate. The labeling pattern in the pheromone is consistent with formation of butyrate from two acetates, followed by α-oxidation and decarboxylation to form the three-carbon acyl unit. The complementary pattern was also observed for the 1-[^{13}C]acetate feeding: among the smaller carbon signals, that for C-3 was more intense than those of C-4 or -10, which is just the opposite from the unlabeled standard. Thus C-3 carried more ^{13}C label, as is expected for this three-carbon unit derived from a butyrate.

The labeling by 1,2,2,2-D$_4$-acetate was not informative. Because acetate is involved in so many metabolic reactions and because the α protons of organic acids are relatively labile, the label became thoroughly scrambled, and the experiment gave no information about pheromone biosynthesis.

Significance to other systems

We anticipate that the similar biosynthetic steps occur for all of the *Carpophilus* hydrocarbon pheromones because of the structural similarities among these and because the labeling of the *C. freemani* minor pheromone components was consistent with the proposed biosynthetic scheme to the extent that analysis was possible. Furthermore, the general scheme is consistent with the

oxygenated polyketide pheromones of other beetle groups. Hydroxyl or carbonyl compounds would be produced instead of hydrocarbons if one or more of the reduction or dehydration steps are omitted.

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